cdt1 is an essential target of the Cdc10/Sct1 transcription factor: requirement for DNA replication and inhibition of mitosis

Johannes F.X.Hofmann and David Beach

Howard Hughes Medical Institute, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA

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We have used an immunoprecipitation−PCR cycle to isolate physically genomic DNA sequences that are bound by the fission yeast cdc10 gene product in an attempt to identify novel target genes. An essential gene, cdt1, has been isolated whose expression is cell cycle regulated in a cdc10 dependent manner. The cdt1 promoter contains a recognition site for a sequence specific DNA binding factor. The cdc10 gene product is a component of this factor. Ectopic expression of cdt1 can complement a temperature sensitive mutation of cdc10 at semipermissive temperature. Cells carrying a null allele of cdt1 are defective in DNA replication but initiate mitotic events, suggesting that cdt1 is essential for the normal dependency relationship of S-phase and mitosis.
Key words: cdc10/cell cycle/start/transcription

Introduction

In the fission yeast, *Schizosaccharomyces pombe*, two genes necessary for the completion of START, *cdc2* and *cdc10*, have been identified by screening *cdc* mutants for those that could conjugate while blocked in cell cycle progression at the restrictive temperature (Nurse and Bissett, 1981). The *cdc10* gene has been cloned by rescue of its mutant phenotype (Aves *et al.*, 1985), but its function was poorly understood until recently. Subsequently it has been demonstrated that the fission yeast *cdc10* gene product is part of a conserved DNA binding complex (Lowndes *et al.*, 1992a). Another fission yeast cell cycle START gene, *sct1*, has been identified as a dominant extragenic suppressor of *cdc10* (Marks *et al.*, 1992; Caligiuri and Beach, 1993). A multicopy suppressor of *pat1* and *cdc10* mutants, called *res1* (Tanaka *et al.*, 1992), is identical to *sct1*. *sct1* is required for commitment to the mitotic cell cycle and p72^{sct1} has been shown to bind to the same cell cycle regulatory DNA sequences as p85^{cdc10} (Caligiuri and Beach, 1993). It has been proposed that this DNA binding activity is involved in the regulation of the cell cycle specific expression of the *cdc22* gene (Gordon and Fantes, 1986; Lowndes *et al.*, 1992b), which until recently was the only known potential target gene. *cdc22* has been identified as the large subunit of ribonucleotide reductase (Fernandez-Sarabia *et al.*, 1993) and loss of its function arrests cells in S-phase. Since loss of *cdc10* function arrests cells at START (Nurse *et al.*, 1976), it is very likely that *cdc10* is involved in the cell cycle regulated expression of a number of genes whose expression is required in the pathway from START to S-phase.

In budding yeast two distinct DNA binding activities are involved in the cell cycle regulation of a large number of genes that are directly or indirectly involved in DNA synthesis (reviewed in Andrews and Herskowitz, 1990; Johnston, 1992; Merril *et al.*, 1992). The product of the *SWI6* gene, which shares sequence homology with *SWI4* and with the fission yeast genes *cdc10* and *sct1* (Breeden and Nasmyth, 1987a; Andrews and Herskowitz, 1989; Tanaka *et al.*, 1992; Caligiuri and Beach, 1993), is required for both activities. It interacts either with SWI4 in the SBF complex (Breeden and Nasmyth, 1987b; Ogas *et al.*, 1991) or with the recently identified p120 in the MBF or DSC1 complex (Dirick *et al.*, 1992; Lowndes *et al.*, 1992a), and binds to two distinct *cis*-acting regulatory elements, SCB and MCB, respectively. MCB is involved in the coordinate regulation of a large number of periodically expressed DNA synthesis genes (Lowndes *et al.*, 1991). The SCB element is found in the upstream region of genes like *HO*, *CLN1*, *CLN2* and *HCS26* (Nasmyth, 1985; Breeden and Nasmyth, 1987b; Nasmyth and Dirick, 1991; Ogas *et al.*, 1991).

SWI4 and SWI6 have been proposed to play an essential role in a positive feedback loop activated at START (Cross and Tinkelenberg, 1991; Nasmyth and Dirick, 1991; Ogas *et al.*, 1991), which also requires the activity of the cell division kinase p34^{CDC28}, and the G$_1$ cyclins CLN1 and CLN2. The function of SWI4/SWI6 is dependent upon START (CDC28) (Breeden and Nasmyth, 1987b), and although *swi4* or *swi6* mutations are not lethal individually, cells simultaneously disrupted for *swi4* and *swi6* are inviable. The lethality of the double mutant can be rescued by a plasmid that expresses *CLN2* from a strong constitutive promoter. Thus, it has been suggested that the lethality of the double mutant is due entirely to a defect in the expression of G$_1$ cyclins. *CLN2* appears to be a target gene of SWI4/SWI6 and acts downstream of the latter (Nasmyth and Dirrick, 1991), but also has been proposed to encode a component of the G$_1$ specific form of the CDC28 kinase (Wittenberg *et al.*, 1990). It is poorly understood, however, how this presumptive autostimulatory feedback loop is activated, what its output signals are, and how it is turned off.

In order to understand better the events that lead to the initiation of DNA synthesis in fission yeast, we decided to isolate potential target genes of *cdc10*. Here we report the identification and characterization of a cell cycle regulated target gene, *cdt1*, which is required both for DNA replication and presumably in a pathway that inhibits premature initiation of mitosis.

Results

Isolation of genomic binding sequences
We have used a cyclical procedure of immunoprecipitation and amplification of genomic DNA to identify and clone specific genomic target sequences of the presumptive transcription factor *cdc10* (see Materials and methods). Conditions for the immunoprecipitation were originally
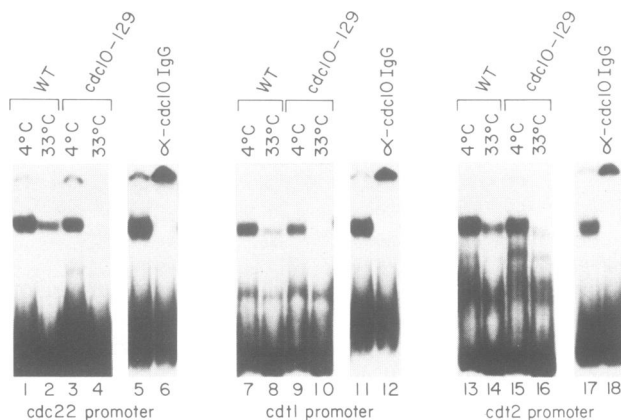
Fig. 1. Characterization of a DNA binding activity that physically contains the *cdc10* gene product. Binding to the isolated genomic binding sites. Subcloned and end-labeled PCR fragments were used for gel retardation assays. Extracts were prepared at 4°C either from wild type (lanes 1, 2, 5–8, 11–14, 17 and 18) or from SP25 (lanes 3, 4, 9, 10, 15 and 16) and incubated in binding buffer at the indicated temperatures before the addition of DNA. 1.4 μg of purified IgG was added to a binding reaction at 4°C (lanes 6, 12 and 18).

developed using a triple *Mlu*I element. This element has been shown to confer cell cycle dependent expression in late G₁ on a heterologous gene and is recognized by a DNA binding activity from fission yeast cell extracts that contain the *cdc10* gene product (Lowndes *et al.*, 1992a).

In order to isolate presumptive target sequences of this DNA binding activity, total genomic DNA from *S.pombe* was digested with *Sau*IIIA and ligated to specially designed adaptors to allow subsequent PCR amplification and cloning. The genomic DNA was then incubated with whole cell extract and immunoprecipitated with anti-p85^{cdc10} antibodies. The precipitated genomic DNA was subsequently amplified by PCR using the appropriate adaptor as a primer (see Materials and methods). After three rounds of sequential immunoprecipitation and PCR, the specifically amplified DNA fragments were cloned. Twelve clones were isolated and eight of them were demonstrated to map to different sites in the genome using Southern blot analysis (data not shown). These eight clones were end-labeled and further analyzed using gel retardation assays. Three examples are shown in Figure 1. All clones were recognized by a specific DNA binding activity that is temperature sensitive in extracts prepared from a strain that carries the temperature sensitive *cdc10*-129 allele (Figure 1, lanes 4, 10 and 16). Also, the specific complexes could be supershifted by antibodies directed against the *cdc10* gene product (Figure 1, lanes 6, 12 and 18). Taken together these results confirm the specificity of the employed isolation procedure.

Characterization and cloning of target genes

Since the identified binding sites potentially represent regulatory promoter sequences of the respective target genes we screened genomic libraries, using the isolated genomic PCR fragments as hybridization probes. Several overlapping genomic clones were isolated with each individual probe. Coding sequences that were present on the genomic clones were then used to isolate the corresponding cDNA clones. If these clones represent the hypothetical target genes one would assume that their expression is regulated by *cdc10*
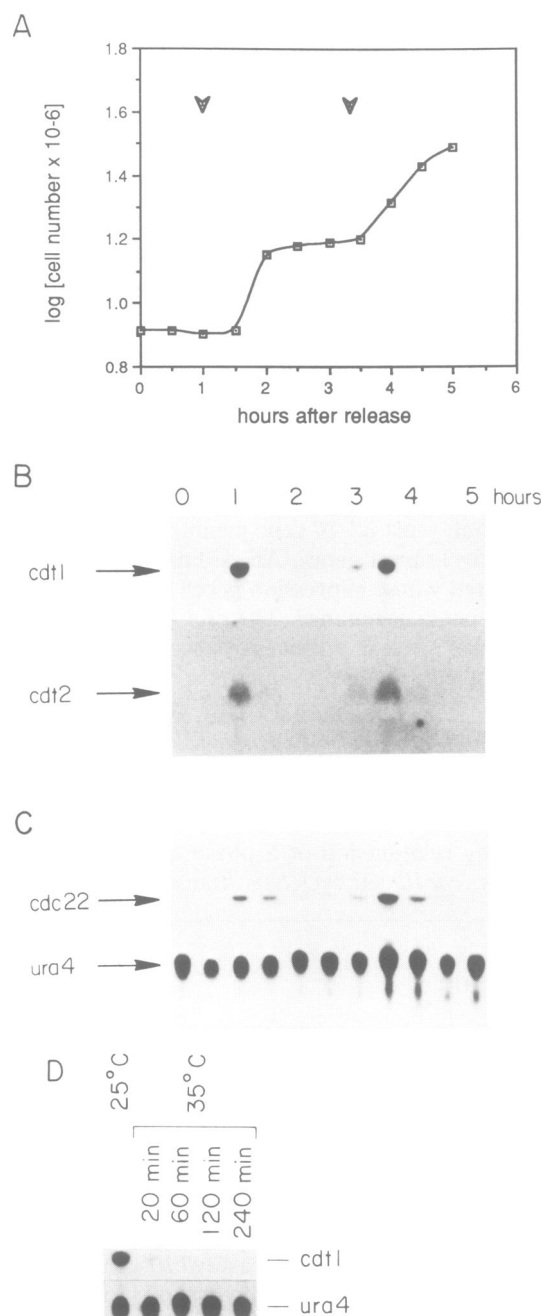

Fig. 2. Cell cycle dependent expression of *cdt1* and *cdt2*. (A) Cell number increase of a synchronous culture. Cell cycle progression was blocked at the G₂/M boundary by incubating a *cdc25*-22 cell culture in exponential growth phase at 35°C for 4 h. The temperature was shifted to 25°C at time 0 and samples were taken every 30 min for cell number counting and RNA isolation. (B) Northern blot analysis of RNA isolated from the synchronous culture. Total RNA was isolated and separated on a 1% agarose gel containing formaldehyde. After blotting on to nylon filter, hybridization was done with random-prime labeled probes of the indicated genes. The expression of *cdt1* and *cdt2* is shown following two consecutive synchronous divisions. Peaks in steady state levels of mRNA of *cdt1* and *cdt2* are indicated by arrowheads in A. (C) The same Northern blot as shown in (B) was reprobed to examine the expression of *cdc22*. The constitutive expression of the *ura4* gene was used to control for loading. Autoradiography was done at −70°C with intensifying screen for 1 day except for *cdt2* for which exposure was done for 7 days. (D) *cdt1* transcript levels were followed by Northern blot analysis after shifting *cdc10*-129 cells from the permissive (25°C) to the restrictive (35°C) temperature. The time points after the temperature shift are indicated at the top. Shown are *cdt1* transcripts and constitutively expressed *ura4* transcripts which were used as a loading control.

A.

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GTTTTTCTATTTGTACTTGGTTGATTTCAATCTGGTCCTTATAGATATTTGAGTCAC
ATTGTTACTCTACCCAGTATAAATAGAAAAGACTTAGTCGATAGAAATCATACAG
GGCATCTTTTTATTCATTTATTATAGCAACTTATCCACTGATAGTAAAAAAGTTA
                EcoRI      **
ACTAAAAGTTTTGAATTCGTACAACTTATGGAAGACCATAAGTGGACGAGTGGCT
ATTTGACACAAGTTTGTTTTCAGAGAGCCTGAACTTGGTGCAACACCTTTATTATA
CAGAAATCATTGTGACATGTAACATATTATTGAAAATTTCGACAACGAACAACTC
   -302                      -282                 -257
AATAATATCTCGCAGGCAAAGGAGTTATTCAAAATTTATATAACGATGCATCCTG
TAATAATTTTAAACTCGTATAAGAGATTCTATAAAAACTTCGGAAAATGAAAAGC
TGTAGTTAAAAAATTGTTTTGTGAATTTTGTACAAGTGTTGGAACTTATTTATTCA
ATATAATCTACTGAATCATATAGGCTAAGTAAGACATAAACTGATTAGTCGGACA
                                   MluI
AATGTGAACTAGTTAGCAAGCGCGACACGATAGCTACGCGCACGCGTCAACAAACGC
                                       +1
GACACGCTCAAATTTAATATAACCCTCGATATAAATG
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Fig. 3. The *cdt1* gene. (A) *cdt1* promoter. 650 bp genomic sequence of the promoter region of *cdt1* are shown. The A residue of the predicted initiator methionine serves as reference for the numbering and is indicated as +1. Nucleotides −257 to −282 are underlined and represent the protected sequence as derived from Figure 4. Other weakly protected regions are indicated by the italicized and underlined sequences. The two stars indicate the 5' end of the initially isolated PCR promoter fragment. The *Mlu*I and *Nhe*I sites were used for the construction of the null allele of *cdt1* (see Materials and methods). The *Nhe*I site is shown in (B). (B) *cdt1* coding sequence. The nucleotide sequence and predicted amino acid sequence derived from the isolated cDNA are shown. Nucleotide numbers are given on the right and amino acid numbers on the left.

B.

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1    ATG AAA TGT CGC GCC CTA TAT AGT CAC GAC AAC AAT ATC CTT ATT TTT GGA TTT  54
     M   K   C   R   A   L   Y   S   H   D   N   N   I   L   I   F   G   F

     TGT GTT CAA ATC ATG AGC GCT GGA TCG CAA ACA AAA TTG AAC TTC TCT GTC CGT
     C   V   Q   I   M   S   A   G   S   Q   T   K   L   N   F   S   V   R

37   AAA ACT CGA TCC TCA TTA AAA AGG TCG AAT GCG GCC ATT ATT GAG CCA CCC AAA 162
     K   T   R   S   S   L   K   R   S   N   A   A   I   I   E   P   P   P   K

     AAT CCT GAG GAT TCT CAA ATA ATA CCA GCT GTT AAA AGG CTG AAA GAG AAC TTG
     N   P   E   D   S   Q   I   I   P   A   V   K   R   L   K   E   N   L

73   GAC ACT GAG TCT TTA GAA CAA AAT GAG GTT TTA CCC CCA GTT AAA AAT GAA TCA 270
     D   T   E   S   L   E   Q   N   E   V   L   P   P   V   K   N   E   S

     GTT CTT TTC TTA GAA AAG GTC TTT AAT GCA GTC GAC ATA TGT GTA AAA TTT CAC
     V   L   F   L   E   K   V   F   N   A   V   D   I   C   V   K   F   H

109  CTC TCC ATC AAT ACC AAA CCA ACC TTT GTG CTG TTA GAA AAT AAA GTC TCT GGT 378
     L   S   I   N   T   K   P   T   F   V   L   L   E   N   K   V   S   G

     CTC ACC AAA ATC TCC TTA AAA ATA ACT CAT CTC GCT CAG ATT TTA ACC GTA TGG
     L   T   K   I   S   L   K   I   T   H   L   A   Q   I   L   T   V   W

145  CCA GAG TCA TTT GCT ATT ACC CCA TGT TTT ACT ATT CAT CAA GGA AAG CGT GTT 486
     P   E   S   F   A   I   T   P   C   F   T   I   H   Q   G   K   R   V

     GCA ACA TAT GAA CTG TCA TAC CCT CGC AAT GCG AAT CTT CCT GAA GCC TTC TCC
     A   T   Y   E   L   S   Y   P   R   N   A   N   L   P   E   A   F   S

181  CGC TCC ATT GAA TTT AAA CGG AGA CTA GAA AAA TGG TTG TTA GAG CAT TGT TCT 594
     R   S   I   E   F   K   R   R   L   E   K   W   L   L   E   H   C   S

     GAA ACT GAG ATT CCT GCT CAA CAG TTA CAA GCT TTA CCC AGT CTA TCC AAG AAT
     E   T   E   I   P   A   Q   Q   L   Q   A   L   P   S   L   S   K   N

217  ACT GTT AAT GAA AGT TCA CTA GTT CGA AAG CTC AAT TTG GAG AAA TCA ACA AGT 702
     T   V   N   E   S   S   L   V   R   K   L   N   L   E   K   S   T   S

     CGC GAG TTA CGT ATT CCC ACA CAA ACT CTA GAA CCA AAA TTC ACT ACT AAC ACG
     R   E   L   R   I   P   T   Q   T   L   E   P   K   F   T   T   N   T

253  GCA AAA TAT GCT AAT GAG TTA GTC TCG TGT AGC ATG CTT GAC AGT TCG TCC ACT 810
     A   K   Y   A   N   E   L   V   S   C   S   M   L   D   S   S   S   T

     TTG TCA AAG TCA GTG AAT TCA AAA ATC AAT TTG AAA TCC CAT CAA AGT TCT TCA
     L   S   K   S   V   N   S   K   I   N   L   K   S   H   Q   S   S   S

289  TCT GTT CAA AAT TCA TCG CGT AAG CTA ACA TCA TCT CAG TTA ACT TTG AGA CAA 918
     S   V   Q   N   S   S   R   K   L   T   S   S   Q   L   T   L   R   Q

     TCT TCT CTG TTC GAT CGC GTG CGT AAA AAG CAA AAA GCA ATG GAA GCA AAA AAA
     S   S   L   F   D   R   V   R   K   K   Q   K   A   M   E   A   K   K

325  GCT GAA GAA TTT AAA AAC AAC CTA GTT GTT CAT ACT CTA GCA AAA GAA AAG GTC 1026
     A   E   E   F   K   N   N   L   V   V   H   T   L   A   K   E   K   V

     TCA TTT GTC CGC ATT ATT GAT CTC ATT TTT GTT CAG CTT TCC ACT TGG CCA ACT
     S   F   V   R   I   I   D   L   I   F   V   Q   L   S   T   W   P   T

361  AAA CGA TCT TTT TCT ATG TCT GAA ATT GTG ACA AGC ATG CAA ATG TCG ATA TCA 1134
     K   R   S   F   S   M   S   E   I   V   T   S   M   Q   M   S   I   S

     TCA AGC TTA TCA TCT GAC CAG TGT GCC AAA GCA ATT GAG TTG CTT TCA AAA GCT
     S   S   L   S   P   D   Q   C   A   K   A   I   E   L   L   S   K   A

397  TTG CCT GCT TGG TGC ACT ATC AAT CTT CTT GGT AAT ATT CAA GTA GTC ACA TTC 1242
     L   P   A   W   C   T   I   N   L   L   G   N   I   Q   V   V   T   F

     AGC AGG ATT GTT AAT GGG AAA CCA TAC TTG CGA TCC CAA TTA ATT GAA GAA TTG
     S   R   I   V   N   G   K   P   Y   L   R   S   Q   L   I   E   E   L
                        NheI
     CAA ACA AAG GCT AGC ATC ACT ATT CTT TCA AAT TCT TAA
433  Q   T   K   A   S   I   T   I   L   S   N   S
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at the level of transcription in a cell cycle dependent manner, and that their expression is altered in mutants defective in the *cdc10* gene.

To test these predictions, the expression of these genes during the cell cycle was investigated using synchronous cultures. Synchronization was achieved by shifting mutant *cdc25-22* cells (strain SP32) to the restrictive temperature for 4 h to block cell cycle progression in G₂, followed by release to the permissive temperature. The following two synchronous divisions were examined (Figure 2A). Total RNA was isolated at 30 min intervals and equal amounts were loaded on formaldeyde gels for Northern blotting. Random primed cDNA probes were used for hybridization; three examples are shown in Figure 2B and C. We identified two novel genes (*cdt1* and *cdt2*; for *cdc10* dependent transcripts 1 and 2) whose steady state RNA levels are subject to cell cycle regulation. Transcript levels of the two genes peak significantly during G₁ of the two synchronous divisions and are almost undetectable during the other phases of the cell cycle (Figure 2B). The third gene identified was found to be identical to the *cdc22* gene, whose transcript has previously been shown to be cell cycle regulated reaching maximum levels during late G₁/S (Gordon and Fantes, 1986; Figure 2C). Although transcripts of the three genes appear with the same kinetics in the early phase of the cell cycle, *cdc22* transcripts persist longer than those of *cdt1* and *cdt2*. The reason for the different regulation of these transcripts is not known and might involve different regulatory transcriptional complexes, different promoter elements or different stabilities of transcripts.

We also looked at steady state RNA levels of the identified genes in cells that had been blocked in G₁ using the temperature sensitive allele *cdc10*-129: total RNA was isolated from an SP25 culture in exponential growth phase that had been shifted to the restrictive temperature. Transcript levels of *cdt1* dropped significantly within 20 min at the restrictive temperature (Figure 2D). It appears that normal expression of *cdt1* in G₁ of the cell cycle requires a functional *cdc10* product and that the expression of *cdt1* is severely affected *in vivo* by a nonfunctional *cdc10* product. The abundance of *cdt2* RNA did not change significantly under these conditions (data not shown), suggesting that basal levels of expression are sufficient for the production of significant amounts of transcripts, or that other factors are also involved in the expression of *cdt2*.

These observations suggested that *cdt1* may be a rate limiting target of the *cdc10* transcription factor; we therefore decided to analyze the *cdt1* gene further. A full-length cDNA clone was isolated using the genomic clone as hybridization probe. The isolated 1556 bp cDNA contains a single open reading frame of 444 amino acids (Figure 3B). No significant sequence similarities to any known proteins or any known sequence patterns have been found using the predicted protein sequence of *cdt1* and the FASTA program (Pearson and Lipman, 1988) in a screen of the GenBank databases. The *cdt2* gene is the subject of a separate study.
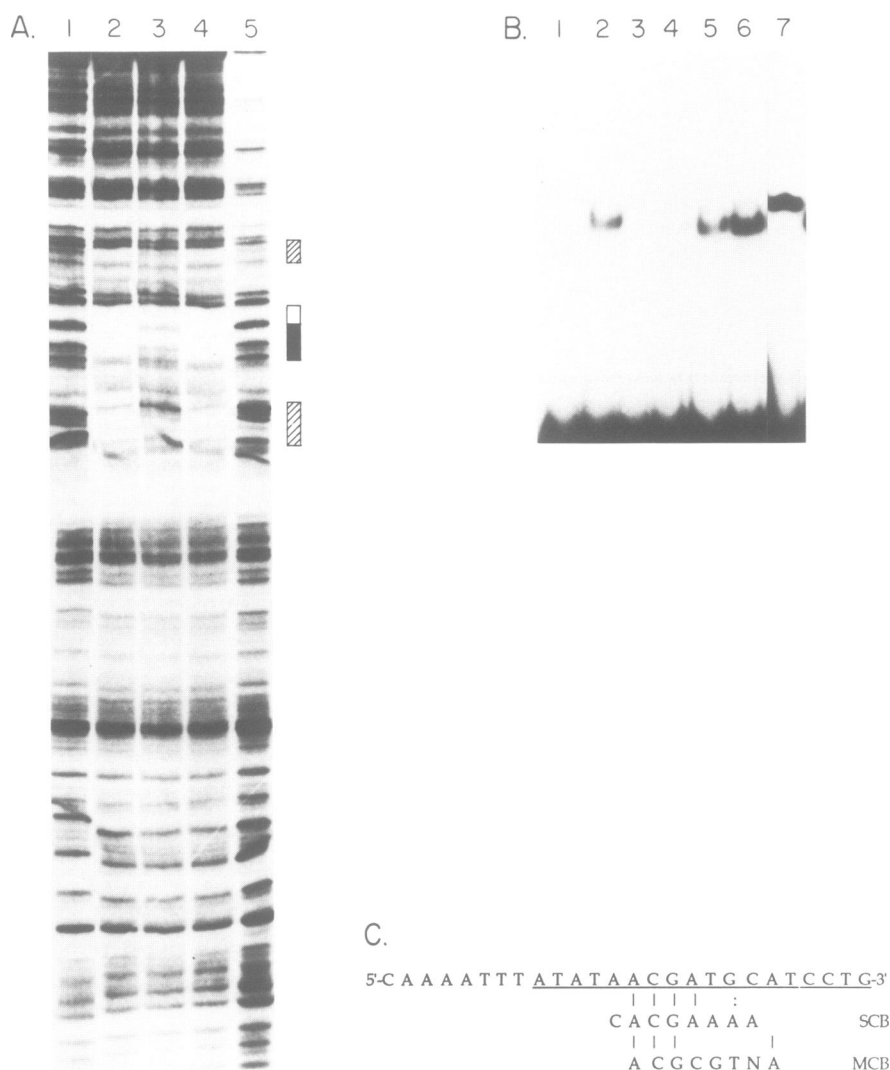
Fig. 4. Mapping of a promoter binding site. (A) DNA footprint analysis. A 414 bp EcoRI−MluI fragment from the cdt1 promoter was used for protection studies. Naked DNA was digested with different amounts of DNase I (lanes 1 and 5). Closed bars indicate protection from enzymatic cleavage in the presence of whole cell extract (lane 2). The 5' border of the protected sequence could not be unambigously assigned since no cleavage could be detected in this area on naked DNA (open bar). Short regions with apparently reduced cleavage are indicated by striped bars (see also Figure 3A). Competition was done using a 50-fold molar excess of the triple MluI element or a mutated version (lanes 3 and 4, respectively). (B) The double-stranded end-labeled oligonucleotide (5'-TTCAAAATTTATATAACGATGCATCCTGTA-3') was used for a gel retardation assay. The oligonucleotide was incubated with whole cell extract (lanes 2−5). The triple MluI element or the mutated MluI element were used as competitor at a 100-fold or 10-fold molar excess (lanes 3 + 4, and 5 + 6, respectively). To one reaction 1.4 μg of purified α-cdc10 IgG was added (lane 7). (C) An alignment of the derived binding site with the SCB and MCB elements from S.cerevisiae is shown (the underlined sequence corresponds to the closed bar in Figure 5B). Identical residues are indicated by vertical bars; a purine residue at the same position is indicated by a colon.

The cdt1 promoter-binding activity

In order to establish further whether cdt1 is a direct target gene of cdc10 we attempted to map DNA binding activities on the cdt1 promoter. A 415 bp genomic EcoRI−MluI fragment of the cdt1 promoter (Figures 3A and 4A) was used for DNA footprint analysis. Several regions on this fragment were found to be cleaved with reduced efficiency after incubation with whole cell extract and treatment with DNase I. The most prominent protection spans 26 nucleotides between −282 and −257 bp upstream of the predicted initiator methionine [Figures 3A, 4A (lane 2) and 4C]. This binding activity is specific since it can be competed by the triple MluI element but not by the mutated version (Figure 4A, lanes 3 and 4). To determine if the protected sequence between nucleotides −282 and −257 is sufficient for binding, a synthetic double-stranded oligonucleotide that

contains the protected sequence was used in a gel retardation assay (see Materials and methods). Specific complex formation was detected in this assay, which can be competed by the triple MluI element or the 415 bp cdt1 promoter fragment (Figure 4B, lanes 3 and 4, respectively); the mutated triple MluI element does not compete (Figure 4B, lanes 5 and 6). These experiments suggest that nucleotides −282 to −257 of the cdt1 promoter are probably involved in binding of an activity that is indistinguishable from the MluI binding activity.

In Saccharomyces cerevisiae the two related cell cycle START dependent transcription factors SBF and DSC1 bind to promoters of a number of cell cycle regulated genes that are required either for the G_1/S transition or DNA synthesis. The recognition sequences of these factors are the 8 bp motifs MCB and SCB. An alignment of the protected
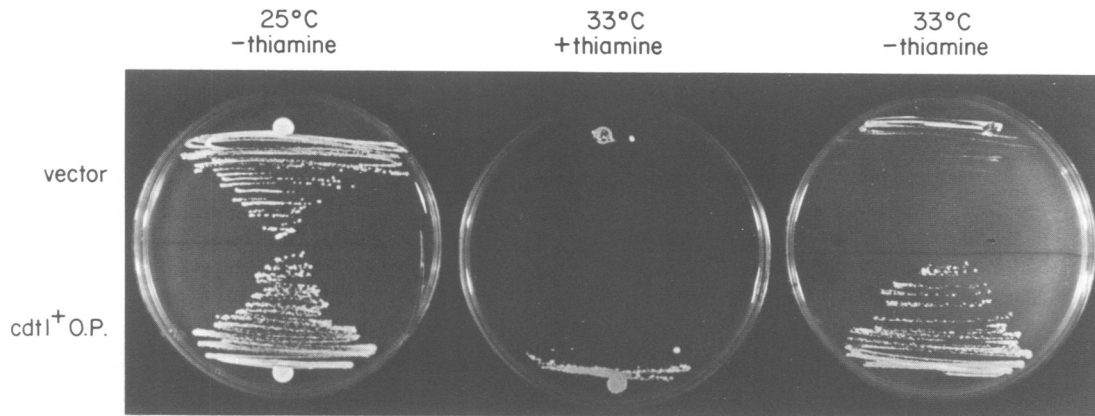
Fig. 5. Complementation of *cdc10*-129 by ectopic expression of *cdt1* at semipermissive temperature. Strain SP39 was transformed with a vector that expresses the *cdt1* gene from the modified *nmt1* promoter, pREP81 (Basi *et al.*, 1993), or with the vector alone and colonies were restreaked on PMA plates under the indicated conditions. Ectopic expression of *cdt1* from this promoter allows colony formation in a *cdc10*-129 background at 33°C in the absence of thiamine (inducing conditions) but not in the presence of thiamine (repressing conditions).

sequence from the *cdt1* promoter with these two motifs is shown in Figure 4C. The primary structures of the three motifs display some limited sequence similarity (see Discussion), which possibly reflects the evolutionary conservation of positions that are important for recognition by functionally and structurally related complexes.

Ectopic expression of cdt1 complements a cdc10⁻ temperature sensitive mutant

As shown previously, mutant *cdc10*⁻ cells arrest cell cycle progression in G_1 prior to the initiation of DNA synthesis (Nurse *et al.*, 1976; Marks *et al.*, 1992). It is possible that under restrictive conditions in *cdc10*⁻ mutant cells certain target genes are not expressed at a sufficient level, leading to cell cycle arrest. Therefore, ectopic expression of target genes from a heterologous promoter should complement *cdc10*⁻ mutants. To test this hypothesis, we transformed the mutant strain *cdc10*-129 (SP39) with a plasmid that expresses the *cdt1* gene from the thiamine repressible promoter *nmt1* (Maundrell, 1990, 1993). Transformants were selected at 25°C and tested for the ability to form colonies at various elevated temperatures. Whereas the transformants could not grow at 35°C, they were able to form colonies at 33°C in the absence of thiamine (Figure 5, bottom part of each plate). In the presence of thiamine, which represses the *nmt1* promoter, growth was very poor at 33°C. Mutant cells that have been transformed with the parental vector alone were not able to form colonies either at 33°C or 35°C (Figure 5, top part of each plate).

Although *cdt1* cannot fully complement *cdc10*-129 at the restrictive temperature, it does so at an elevated temperature (33°C) that would normally not permit colony formation, suggesting that *cdt1* is a target gene of *cdc10* and that its reduced expression in *cdc10*⁻ temperature sensitive mutants contributes to the terminal phenotype in these cells under restrictive conditions. It appears, however, that in addition to *cdt1* the expression of other genes is dependent on a functional *cdc10* product in order to permit cell cycle progression (see Discussion).

cdt1 is an essential gene with dual function

To examine the effect of the loss-of-function phenotype of *cdt1* we replaced the 1.36 kb *Mlu*I − *Nhe*I fragment of the *cdt1* gene with the *ura4* gene. A linear *Eco*RI fragment

containing the disrupted *cdt1* gene was transformed into a diploid strain auxotrophic for uracil (SP818). Stable Ura⁺ transformants were isolated and a 2.1 kb genomic *Hind*III fragment containing 5′-flanking sequences and N-terminal coding sequences of *cdt1* was used for hybridization to *Hind*III digested genomic DNA of the presumptive heterozygous diploid strains. Southern blot analysis of all diploid strains confirmed the replacement of one wild type copy of *cdt1* with *ura4*. After sporulation, the heterozygous diploid produced no viable Ura⁺ segregants in 21 tetrads dissected, indicating that *cdt1* is an essential gene. Presumptive Ura⁺ segregants arrest as single germinated spores. The lethal phenotype of the *cdt1* disruption can be rescued by a plasmid that expresses the *cdt1* cDNA from the *nmt1* promoter. After transformation and sporulation of the diploid strain, haploid Ura⁺ cells can be recovered whose viability depends on the plasmid (data not shown).

In order to characterize the nature of the *cdt1* null phenotype, germination of Δcdt1 spores was followed in liquid culture. Purified spores derived from a heterozygous diploid strain were inoculated into minimal medium lacking uracil. Since ~50% of the spores are uracil auxotrophs and the other 50% carry the replacement of the *cdt1* gene by the *ura4* gene, only those spores that carry the deletion of the *cdt1* locus would germinate. Therefore germinating spores would display the null phenotype. As a control a spore preparation was used from a diploid strain that was heterozygous for a *ura4*⁻ mutation but homozygous at the wild type *cdt1* locus.

The cell number increase in both cultures was monitored during a time course of germination (Figure 6A). In the control culture, cell number began to rise after ~10 h and then increased exponentially. In the Δcdt1 culture cell number did not rise, demonstrating that germinating spores do not complete cell division. To determine the cellular DNA content of the arrested Δcdt1 spores we performed a chemical measurement using the diphenylamine reagent (Figure 6A; see Materials and methods). Initially spores from both cultures had a 1N DNA content. While germinating spores in the control culture began to initiate DNA synthesis at ~8 h after inoculation, the Δcdt1 spores did not duplicate their DNA. The DNA content of germinating spores was also followed during the same time course using flow cytometry. In the wild type control, cells that had completed
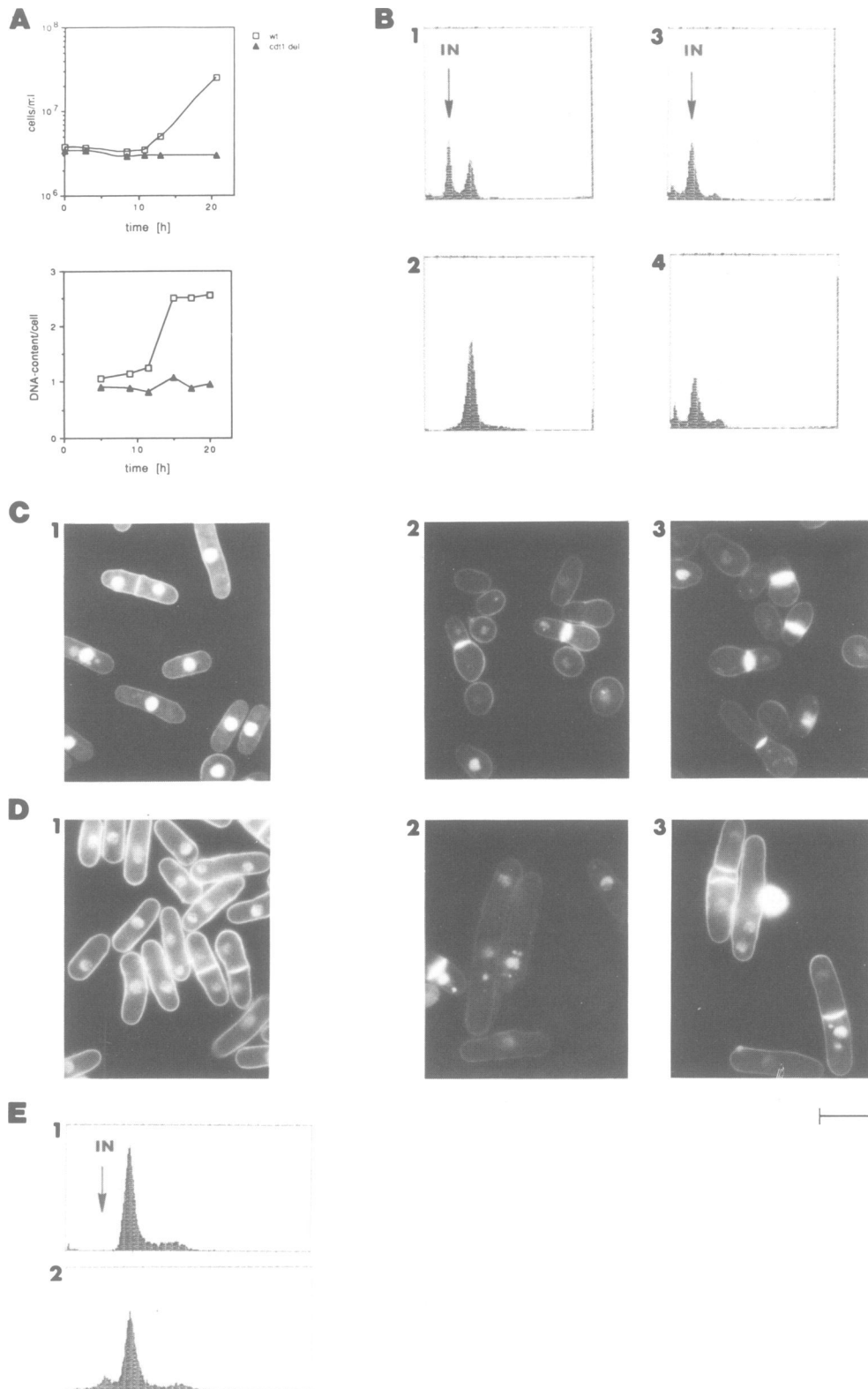
Fig. 6. Phenotype of the *cdt1* null allele. (**A**) Spores were prepared from two different diploid strains: wild type (open squares) are spores prepared from the diploid *h⁻S/h⁺N ade6*-210/*ade6*-216 *leu1*-32/*leu1*-32 *ura4⁻/ura4⁺*. Δcdt1 (closed triangles) are spores from the diploid strain *h⁻S/h⁺N ade6*-210/*ade6*-216 *cdt1⁺/cdt1::ura4 leu1*-32/*leu1*-32 *ura4*-D18/*ura4*-D18. Spores were inoculated at time 0 at 32°C in minimal medium lacking uracil. Cell number was followed in both cultures over a period of 21 h. The cellular DNA content in both cultures was determined using the diphenylamine reaction (see Materials and methods). (**B**) Cell samples from the wild type culture (panels 1 and 2) or the Δcdt1 culture (panels 3 and 4) were taken and subjected to FACS analysis at 8.5 h (panels 1 and 3) and 17 h (panels 3 and 4) after inoculation. (**C**) Panel 1: cells from the wild type culture were fixed with 2% acetaldeyde at 21 h after inoculation and stained with DAPI and Calcofluor. Panels 2 and 3: cells from the Δcdt1 culture were stained as in panel 1. Note that essentially all germinating spores in this culture display the terminal arrest phenotype, which is due to the deletion of the *cdt1* gene. Bar = 10 μm. (**D**) Panel 1: strain SP6 was transformed with pREP81:cdt1, grown in minimal medium and transferred to complete medium for 5 h at 32°C, and cells were then fixed and stained with DAPI and Calcofluor. Panels 2 and 3: strain *h⁻S leu1*-32 *ura4*-D18 *cdt1::ura4* carrying the plasmid pREP81:cdt1 was grown, fixed and stained as in panel 1. (**E**) FACS analysis of the strains described in D1 (panel 1) and in D2 and D3 (panel 2) after 5 h in complete medium.
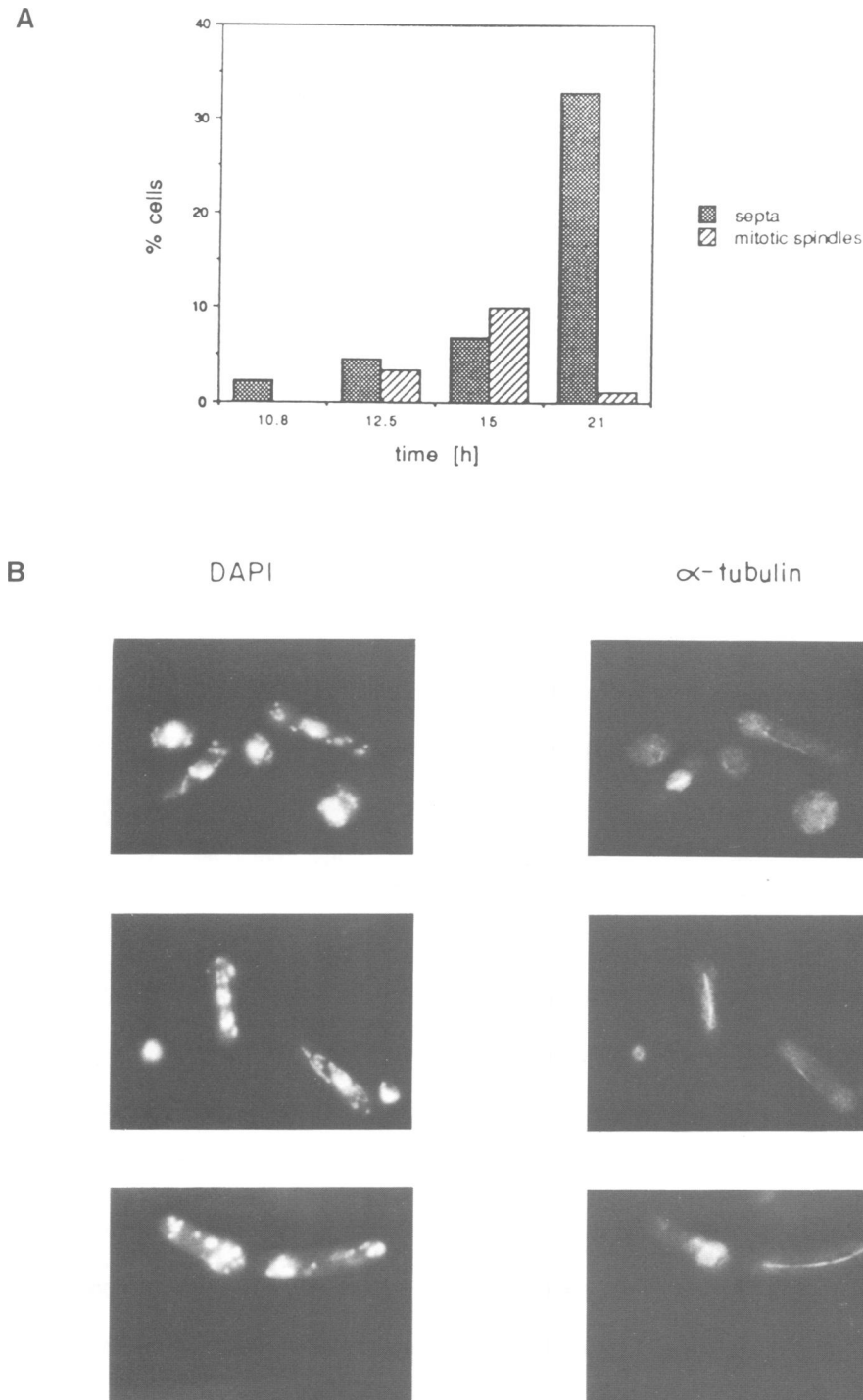
A

B   DAPI   ⍺-tubulin

Fig. 7. Mitotic spindles precede septum formation in Δcdt1 cells. (**A**) Spores from a heterozygous diploid carrying the deletion of *cdt1* were inoculated as described in Figure 6. The percentage of cells containing mitotic spindles or septa was determined at the indicated time points. Note that percentage refers to all spores in the culture and that ~50% of the spores are Ura$^+$ (Δcdt1). At least 150 cells were examined at each time point. (**B**) Cells were fixed and prepared for indirect immunofluorescence at 15 h after inoculation. DNA was stained with DAPI and microtubules were visualized as described in Materials and methods). Three examples are shown. Bar = 10 μm.

DNA replication comprised ~45% of the cells by 8.5 h after inoculation (Figure 6B, panel 1). The exponential growth and division of the Ura$^+$ cells diluted out the non-germinating Ura$^-$ spores which were barely detectable by 17 h after inoculation (Figure 6B, panel 2). In contrast to the control, Δcdt1 spores had not replicated their DNA even by 17 h (Figure 6B, panels 3 and 4); some cell lysis and

clumping became apparent later in the time course (data not shown). These results show that Δcdt1 cells arrest cell cycle progression before the initiation of DNA synthesis or with a defect very early in DNA synthesis.

To investigate the morphology of the cell cycle arrest caused by the deletion of the *cdt1* gene, cells of the two cultures were fixed 21 h after inoculation and stained with

DAPI and Calcofluor to visualize DNA and septum formation, respectively. Ura$^+$ cells in the control culture all germinated and displayed a morphology of normally growing cells (Figure 6C, panel 1). All germinating spores of the Δcdt1 culture, however, arrested cell cycle progression and contained at least one septum. DAPI staining material appeared to be condensed and was found in either or both of the two compartments (Figure 6D, panels 2 and 3). It was surprising that septum formation was detected in the absence of DNA synthesis since septum formation normally requires the activation of mitotic pathways.

The morphology of *cdt1* null mutants was also examined in vegetative cells. A haploid strain with a chromosomal deletion of *cdt1* and transformed with a plasmid that contained the wild type *cdt1* gene was grown under selective conditions and reinoculated into complete medium to allow continued cellular growth after loss of the plasmid. After 5 h in complete medium the culture contained ~30% of abnormal cells (Figure 6D, panels 2 and 3). The most prominent phenotypes were abnormal mitotic structures, septated cells with only one nuclear compartment, and binucleate cells with at least two septa. Fluorescence activated cell sorting (FACS) analysis showed that ~15% of the cells had a 1N DNA content (Figure 6E, panel 2). It should be noted that binucleate cells containing more than one septum are apparently the result of a G$_1$ arrest; however, the two daughter cells are not separated by cytokinesis, because of the aberrant reinitiation of mitotic events; these cells do not contribute to the 1N DNA population detected by flow cytometry. No abnormalities in morphology or cell cycle distribution were found in a wild type strain (SP6) that had been transformed with the same plasmid and grown under identical conditions (Figure 6D1 and E1).

In order to characterize the potential nature of events that lead to the terminal phenotype in more detail, we performed a time course, in which microtuble localization and the formation of septa in germinating spores carrying the *cdt1* deletion were monitored. Appearence of intranuclear mitotic spindles in Δcdt1 cells could be detected at 10 h after inoculation. The spindle index reached its peak at ~15 h (Figure 7A). DAPI staining material appeared to be abnormally separated or fragmented by the mitotic spindle, consistent with the observation that DNA had not been duplicated. No septa were found in cells that contained mitotic spindles, suggesting that these mitotic events were correctly coordinated temporally (Figure 7B). Septated cells accumulated during the time course and by 21 h all germinated cells contained at least one septum (Figure 7A). At this time point, the mitotic spindles were replaced by a weakly staining cytoplasmic array of microtubles, typical of interphase.

These results demonstrate that cells that carry a deletion of *cdt1* induce mitotic events as judged by chromosome condensation, mitotic spindle formation and the formation of septa, despite the fact that they have not replicated their DNA.

Discussion

Here we describe the isolation of target genes of a cell cycle regulated DNA binding complex that contains the *cdc10* gene product. The procedure is an extension of assays that have been developed to identify genomic target sequences of DNA

binding proteins (Kinzler and Vogelstein, 1989; Sompayrac and Danna, 1990; Thiesen and Bach, 1990; Chittenden *et al.*, 1991; Fainsod *et al.*, 1991; Kern *et al.*, 1991; El-Deiry *et al.*, 1992). It involves the isolation of genomic binding sites by a cyclical immunoprecipitation−PCR cycle, followed by the isolation of the presumptive target genes. Yeast represents a very attractive system for this procedure, due to the high gene density and the short distances between regulatory sequences and their respective genes.

The isolated *cdt1* gene is a target gene of p85^{cdc10} based on the following criteria. First, isolated PCR fragments specifically bind to an activity that is temperature sensitive in *cdc10*$^-$ mutants and physically contains the *cdc10* product. Second, *cdt1* is expressed in a cell cycle dependent manner with transcript levels peaking at the G$_1$/S boundary; also, the expression of *cdt1* is dependent *in vivo* on a functional *cdc10* product. Preliminary experiments suggest that a binding site in the *cdt1* promoter is sufficient to confer cell cycle regulated expression to a *lacZ* reporter gene, which is similar to the expression pattern of *cdt1* (data not shown). Third, the ectopic expression of *cdt1* from the heterologous *nmt1* promoter can suppress the growth defects of a *cdc10*$^-$ temperature sensitive mutant at semipermissive temperature.

There is currently no indication of the existence in fission yeast of two distict DNA binding complexes analogous to the budding yeast MBF and SBF complexes. The *cdt1* promoter binding element shows similarity to both the SCB and MCB element. Although no systematic characterization of both elements has been done, the first three positions and the A residue at position 8 of the MCB element have all been implicated in binding to MBF (Dirick *et al.*, 1992) and the G residue at position 3 is essential for transcriptional activation in both budding and fission yeast (McIntosh *et al.*, 1991; Lowndes *et al.*, 1991, 1992b). These positions are conserved in the *cdt1* promoter binding site.

It is not yet understood how the p85^{cdc10} containing DNA binding activity is regulated to confer cell cycle regulated expression of its target genes. In fact the *cdt1* promoter binding activity is present in cell extracts throughout the cell cycle although a slight reduction during mitosis has been observed (data not shown). A similar observation has been made regarding the budding yeast MBF complex, for which no major cell cycle dependent changes in the *in vitro* binding activity to the *TMP1* promoter were found (Dirick *et al.*, 1992). The cell cycle regulated expression of *cdt1* is not an essential requirement for proliferation. Strong ectopic expression of *cdt1* from the wild type *nmt1* promoter has no recognizable effect on the length of generation time in wild type cells (data not shown).

The *cdt1* gene has been shown to be an essential gene. Germinating spores that contain a chromosomal deletion of *cdt1* do not synthesize DNA. However, the normal dependency of M phase on S phase is abolished: mitotic spindle formation and asymmetric segregation of chromosomal material can be observed, followed by cell septation at which point cell cycle progression is arrested. This is consistent with the null phenotype of vegetative cells: cells that carry the chromosomal deletion of *cdt1* are viable when the intact gene is present on a multicopy plasmid; when grown under non-selective conditions, this culture contains a proportion of cells that are aberrantly septated and have anuclear compartments. FACS analysis has shown that ~15% of the cells in this culture have a 1N DNA content, which is consistent with the expected loss rate of the plasmid. It

appears therefore that the loss of *cdt1* results in a defect of an intrinsic checkpoint control mechanism that ensures that mitosis is not initiated before certain events of S-phase have been completed; alternatively *cdt1* may be otherwise required for the dependency of mitosis on S-phase.

Mammalian cell fusion experiments (Johnson and Rao, 1970; Rao and Johnson, 1970) and injection of synchronized HeLa cell extracts into *Xenopus* oocytes (Adlakha *et al.*, 1983) have led to the proposal that dose dependent inhibitors of mitosis are present during the G_1 and S phase of the cell cycle. Negative regulators of mitotic initiation have been identified as mutants that prematurely initiate mitosis under conditions when DNA was damaged or cell cycle progression was blocked before the completion of DNA synthesis (e.g. *rad6*, *bimE* and *hus1-5*; Weinert and Hartwell, 1988; Osmani *et al.*, 1988; Enoch *et al.*, 1992). Other conditional mutations result in premature and lethal mitosis in normally proliferating cells (*wee1-50/mik1*, *pim1* and *spi1*; Lundgren *et al.*, 1991; Matsumoto and Beach, 1991). However, unlike *cdt1*, none of these gene functions identified appear to be required for DNA synthesis in yeast. It has recently been shown that the budding yeast *CDC6* gene product, which is required for DNA replication, can delay the initiation of M-phase in budding and fission yeast when constitutively expressed (Bueno and Russell). Further investigation will be required to establish its normal role in the budding yeast cell cycle. Very recently two fission yeast genes, *cdc18* and *cut5* (Kelly *et al.*, 1993; Saka and Yanagida, 1993), have been shown to be required for the normal dependency of mitosis on the completion of S-phase. In the absence of their normal gene function cells do not synthesize DNA but appear to enter aberrant mitosis resulting in a 'cut phenotype', which is very similar to the one described in this study. Most importantly, like *cdt1*, *cdc18* appears to be an important target gene of *cdc10*. These observations suggest that *cdt1*, *cdc18* and *cut5/rad4* may operate in the same or a similar pathway.

Currently it is not understood what the precise role of *cdc18*, *cdt1* and *cut5* in DNA synthesis is. However, cellular factors that are directly involved in some aspect of DNA replication would be ideally suited as sensors for the completion of events that lead to the commitment to S-phase. The precise role of these genes in the activation of DNA synthesis and potential downstream events that are involved in the inhibition of premature mitotic events remain to be elucidated and will be the subject of future investigation.

Materials and methods

Strains and media
All strains were derived from wild type strains originally described by Leupold (1970). Strains used in this study are listed in Table I. Growth conditions and media were as described by Beach *et al.* (1985).

Cell extracts and gel retardation assay
Cell extracts were prepared by resuspension of washed cells in breakage buffer (50 mM Tris—Cl pH 7.6, 300 mM KCl, 5 mM EDTA, 10% glycerol, 0.2% NP-40, 2 mM DTT, 0.4 mM PMSF, 20 µg/ml leupeptin, 20 µg/ml aprotinin, 0.1 mM sodium vanadate, 20 mM β-glycerophosphate) followed by glass bead disruption. The extract was cleared by centrifugation at 15 000 *g* at 4°C and frozen at −70°C. DNA binding reactions were done at room temperature, unless otherwise indicated, in 50 mM Tris—Cl pH 7.6, 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 10% glycerol, 0.1 mM PMSF. Reactions were loaded on 5% polyacrylamide gels; running buffer was 0.25 × TBE.

Table I. List of *Schizosaccharomyces pombe* strains

Strain	Genotype
SP6	h^{-s} *leu1-32*
SP25	h^{-s} *cdc10-129*
SP32	h^{-s} *cdc25-22*
SP39	h^{+N} *cdc10-129 leu1-32*
SP596	h^{-s} *wee1-50 leu1-32*
SP623	h^{-s} *cdc2-1w leu1-32*
SP818	h^{-s}/h^{+N} *ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18*
SP1154	h^{-s} *cdc10-C4*

Isolation of genomic binding sites
Genomic DNA was digested with *Sau*IIIA and the ends were partially filled in using dGTP and Klenow polymerase to avoid self-ligation in the following steps. Two complementary adaptors were used to tag the ends of genomic fragments, A (5′-GAGTAGAATTCTAATATCTC-3′) and B (5′-ATC-GAGATATTAGAATTCTACTCA-3′). Adaptor B was phosphorylated using T4 polynucleotide kinase, annealed to adaptor A and ligated to the genomic restriction fragments. 150 ng of tagged genomic DNA were incubated in 100 µl 20 mM Tris—Cl pH 7.6, 20 mM KCl, 5 mM MgCl₂, 0.8 mM ATP, 0.1 mM PMSF, 10% glycerol containing 6 mg poly(dIC) and 50 µg whole cell extract for 20 min on ice. 7 µg of a purified anti-cdc10 rabbit IgG were added and the incubation was continued for 15 min on ice. The reaction was brought to room temperature and EDTA was added to a final concentration of 10 mM, followed by 10 µl protein A—Sepharose (50% slurry in wash buffer: 20 mM Tris—Cl pH 7.6, 50 mM KCl, 1 mM EDTA, 0.05% NP-40, 0.02 mg/ml *Escherichia coli* tRNA). The incubation was continued for 15 min at room temperature with gentle mixing, then diluted to 1 ml with wash buffer. Protein A—Sepharose was pelletted and washed three times with 1 ml wash buffer. The pellet was resuspended in 150 µl of 25 mM EDTA, 0.5% SDS containing 10 µg *E.coli* tRNA, and incubated at 68°C for 10 min to release bound genomic DNA. The supernatant was extracted with phenol—chloroform, ethanol precipitated and resuspended in TE. DNA was amplified according to the GeneAmp PCR core reagents kit (Perkin Elmer—Cetus) except that the reaction contained 1% formamide. 30 cycles of amplification were done using adaptor A as a primer and the following temperature profile: 1 min at 94°C, 1 min at 48°C and 1 min at 72°C. The immunoprecipitation—PCR cycle was repeated twice. The specificity of amplification was demonstrated by using purified IgGs from rabbit preimmune serum in a parallel isolation procedure which did not result in the amplification of specific DNA fragments. PCR products were subcloned after digestion with *Eco*RI, for which recognition sequences were present in the flanking adaptor sequences.

DNase I footprint analysis
1 ng of end-labeled DNA was incubated on ice with 1 µg salmon sperm DNA in 25 µl 2 × TM (50 mM Tris—Cl pH 7.6, 12 mM mgCl₂, 1 mM DTT, 20% glycerol, 0.1% NP-40, 0.1 M KCl). 40 µg of whole cell extract were added, the volume was increased to 50 µl and the reaction incubated for 15 min on ice. Then 50 µl of 10 mM mgCl₂ and 5 mM CaCl₂ were added followed by 1 µl of DNase I (20 µg/ml). Digestion was done for 1 min on ice and stopped by the addition of 90 µl of 20 mM EDTA, 1% SDS, 0.2 M NaCl. DNA was phenol extracted, ethanol precipitated and analyzed on a 6% sequencing gel.

Cloning and disruption of cdt1
Using a subcloned PCR fragment from the screen described above, a genomic clone of *cdt1* was isolated from a library containing partial genomic *Sau*IIIA fragments in the *Bcl*I site of pWH5 (Wright *et al.*, 1986). A 6 kb *Sma*I—*Pst*I fragment was released from the vector and subcloned in Bluescript pSK(+) digested with *Xba*I (filled in with Klenow polymerase) and *Pst*I. The internal *Mlu*I—*Nhe*I fragment from this plasmid was then replaced by a 1.8 kb *ura4* fragment. A 4 kb *Eco*RI fragment from this construct containing the *ura4* gene and genomic flanking sequences from the *cdt1* gene was purified and transformed into the diploid strain SP818. Nine stable Ura⁺ transformants were isolated and Southern hybridization showed that all transformants contained one wild type and one disrupted *cdt1* gene. To examine the null phenotype of *cdt1*, one transformant was streaked on sporulation medium and the resulting azygotic asci were dissected on YEA plates. Of 21 dissected tetrads, no tetrad yielded more than two viable spores. All cells descending from viable spores were Ura⁻, indicating that *cdt1* is an essential gene.

The chromosomal map position of *cdt1* was determined by hybridization to an immobilized ordered cosmid bank. *cdt1* maps to the left arm of chromosome 2 between *cdc13* and *mei3*. It has also been excluded that *cdt1* is identical to *cdc18*, *cdc19* or *cdc20*.

Other methods

DNA content of cells was done following alkaline lysis as described by Bostock (1970). Spores were purified according to Beach *et al.* (1985). Indirect immunofluorescence staining for tubulin was done according to Matsumoto and Beach (1991) using the monoclonal anti-tubulin antibody TAT-1. FACS analysis was done according to Costello *et al.* (1986).

Acknowledgements

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Note added in proof

The nucleotide sequence data reported here have been deposited in the EMBL Nucleotide Sequence Database under the accession number Z27248.